The use of ozone in controlling microbial growth on alfalfa sprouts during germination.

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Abstract

Minimally processed foods (MPF) such as fresh vegetables are very popular in the diets of many people due to their healthy appeal. Alfalfa sprouts are one of these MPF that has received great attention in recent years due to the prevalence of food poisoning that has been associated with them. In the autumn of 1998, the FDA issued a statement warning consumers of high-risk groups to avoid consumption of sprouts due to the potential health hazard that they present. Ozone has been used as a disinfectant in water treatment for almost a century. Ozone is one of the strongest oxidizing agents known and will readily form H_2O_2, superoxide, and $HO^{\cdot}$ radicals in the presence of water and is an effective antimicrobial agent. The aim of this study was to determine the effectiveness of using ozone as a treatment in reducing the natural flora on alfalfa sprouts grown from seeds. The effectiveness of ozone is limited by physical barriers, its rapid reactivity and its detrimental effect on the product being treated. An ozone treatment was shown to be effective in reducing the natural flora on alfalfa sprouts by about 2 log, to about 10^7 cfu/g. Ozone is a viable alternative to chlorine in the reduction of natural flora on alfalfa sprouts prior to retail sale. The best treatment process was determined to be treatment via addition and subsequent stirring of the sprouts in ozonated water for a period of twenty minutes.

Literature Review

Minimally Processed Foods

In our health conscious society, many individuals consider "fresh" foods (especially fruits and vegetables) to be more nutritious than processed foods. The result is an increase in the consumption of these foods (Dougherty, 1990 and Ronk et. al., 1989). Minimally processed foods (MPF) is a general description of a broad category of food products that in general are not heated and are not subject to any form of a detrimental processing. Though fresh foods may be better for one's health in many ways, they may also introduce an often-overlooked hazard involving pathogenic microorganisms. This hazard is present due to the lack of processing steps designed to kill, inhibit or reduce populations of harmful microorganisms. That which makes these foods appealing nutritionally also allows for the potential presence and growth of pathogenic microorganisms.

Perhaps the most popular category of MPF are vegetables, which are commonly eaten raw. Several studies have enumerated microbiological prevalence on a variety of raw vegetables. The use of vegetable sprouts in foods has become increasingly popular in recent years. Many varieties of sprouts can be purchased in most supermarkets, varieties include alfalfa, radish, onion and a variety of bean sprouts. Home sprouting kits are even available, enabling consumers to produce fresh sprouts in their homes. Most often the sprouts are eaten raw, or after mild heating and often only a moderate rinsing with water will occur before consumption. The sprouts are grown in a warm, moist environment, which is very favorable for the growth of microorganisms (Prokopowich and Blank, 1991). Such favorable conditions for bacterial growth coupled with the minimal processing make the potential of a sprout-related outbreak greater than many other foods. Therefore, it is not surprising that sprouts were linked to several foodborne disease outbreaks in recent years. Recently the FDA issued a statement warning consumers of high-risk groups to avoid consumption of sprouts due to the potential hazard that they present. This reaction by the FDA followed a disease outbreak in the summer of 1998, which were linked to the consumption of sprouts.

Microorganisms found on Sprouts

Many groups have studied the prevalence of microorganisms in minimally processed foods, including sprouts. Harmon et al. (1987) sampled 98 units of sprouts and found *Bacillus cereus* (a pathogen) to be present on 57% of the samples. Patterson and Woodburn (1980) and Splittstoesser et al. (1983) conducted studies and found that total aerobic plate counts on sprouts range from about 1×10^8 to 4.9×10^8 cfu / gram, and total coliforms were found at populations ranging from 2.0×10^6 to 1×10^7 cfu / gram. Coliforms counts are typically used to evaluate the potential of the presence of more serious organisms such as *E. coli* O157:H7. Much of the contamination of sprouts is believed to come from the seeds themselves and not the growth medium. Splittstoesser et al. (1983) determined that the aerobic plate count of the seeds prior to germination was approximately 10^6 cfu / gram. In another study conducted by Andrews et al. (1982), mold was found on 98.1% of bean sprouts that were not treated with a surface disinfectant.

The conclusions of several studies indicate that washing of fresh produce with water was ineffective in greatly reducing the numbers of microorganisms on the product. The results of two studies showed that only a one-log reduction in the numbers of bacteria present on lettuce could be achieved by washing with water. With such large bacteria populations still present on the produce, bacterial cells could multiply very quickly, if given the opportunity, giving rise to potentially hazardous bacterial populations. Results of studies have also shown that sprouts are an excellent growth medium and the availability of simple sugars and essential amino acids (used by the bacteria during growth) increase during the germination of the sprout (Andrews et al., 1982).

Food Poisoning and Sprouts

Although high concentrations of pathogenic bacteria are a rare occurrence on sprouts, pathogenic bacteria were detected on sprouts in several studies and in several outbreaks. Some of the pathogens found were: *Salmonella, Listeria monocytogenes, Bacillus cereus* and *Escherichia coli* O157:H7 (Beuchat, 1996 and Briley, 1997). Several foodborne illness outbreaks have been traced to sprouts as the source of the pathogenic bacteria. One outbreak, involving *Bacillus cereus*, was traced to sprouts grown in home sprouting kits (Portnoy, 1976).

In the spring of 1988, an outbreak of *Salmonella* Saint-paul located in southern England and Sweden was found to be caused by the contamination of bean sprouts (O'Mahony, 1990). Recently two outbreaks have occurred, both involving *E. coli 0157:H7.* One case occurred in Japan in the autumn of 1996, and the other occurred in Michigan in the spring of 1997 (Anonymous, 1997 and Briley, 1997). Three outbreaks in 1998 involving alfalfa sprouts lead to the FDA warning to high-risk individuals. These cases have exposed the potential hazards associated with vegetable sprouts and fresh produce in general.

Use and Activity of Ozone

Ozone is a water-soluble, blue gas that has been used as a disinfectant in water treatment for almost a century. It is produced in the outer atmosphere by UV radiation and can also be produced by electrical discharge. It is approved as Generally Recognized As Safe (GRAS) for bottled water and has been commonly used in the European food industry for years. Oxidation products produced by ozone are similar to normal oxidation products produced in the body (Graham, 1997). It will decompose readily in air to form free oxygen. The oxidative activity of ozone is dependent on many factors including relative humidity, temperature, pH and organic matter (ozone demand). One of the most important limiting factors is a high ozone demand, which is common in the presence of organic matter. Organic matter may consume ozone or its products before the antimicrobial activity can occur. Ozone is one of the strongest oxidizing agents known (1.5 times greater than chlorine) and will readily form H_2O_2, superoxide, and $HO^{\cdot}$ radicals in the presence of water (Hoigne and Bader, 1975). These intermediates are quickly consumed and are the basis of the antimicrobial activity of ozone. Due to ozone's oxidative potential, many components of foods would be adversely effected by the activity of ozone in the form of oxidized lipids, oxidized proteins and inhibitions of proteins. Unlike chlorine, however, ozone does not lead to the formation of toxic materials in the presence of ammonia or phenolic compounds.

Ozone has been shown to be very effective as an antimicrobial agent in many studies and usually acts quicker than many other antimicrobial agents at lower concentrations and has been shown to be inhibitory towards a broad range of microorganisms, including vegetative organisms, spores, and both Gram-positive and Gram-negative bacteria. Ozone is bactericidal

and is thought to have two primary actions in its bactericidal effect (Venosa, 1972 and Victorian, 1992). The first involves the oxidation of polyunsaturated fatty acids to acid peroxides in membranes and the second involves the oxidation of sulfhydryl groups. It is thought that ozone may diffuse into phospholipid membranes and once within the outer barrier, it can attack the vulnerable unsaturated lipids causing the formation of secondary radical products. These newly formed radical products can react further, ultimately leading to the disruption of the membranes and cell death by lysis. The observed increase of cellular components into the aqueous media, presumably a result of the cell lysis and release of internal contents, supports this model of ozone attack. Investigation of this type of ozone attack is ongoing. Many studies have investigated the inactivation of proteins and enzymes as a result of the bridging of reactive sulfhydryl groups (SH- to S-S) (Barron, 1954). Other studies have suggested that the cell's RNA may be damaged, causing disruption to the cell's biochemical activities (Roy et al., 1981). The debate is ongoing, and recent research has suggested that the actions of $HO^{\cdot}$ radicals alone are not entirely responsible for the inactivation of microorganisms (Graham, 1997, Victorian, 1992). However, there is strong belief that the primary attack of the ozone is on the double bonds of the lipid membrane of microbial cells, resulting in cell lysis (Hewitt and Terry, 1992 and Scott and Lesher, 1963).

There is also strong indication that the presence of water facilitates the inactivation of microorganisms. It has been proven that increased humidity greatly increases the activity of ozone, most likely due to the formation and propagation of free radical products, which ultimately lead to interference in the cell's activity in a variety of mechanisms (Ewell, 1946). Desiccated cells have been reported to be more resistant to ozone attack.

The greatest challenge in using ozone as an antimicrobial agent is that it dissipates quickly in aqueous environments and may not find its way to the microorganisms targeted for control. There are two primary reasons for this, first, organic components (which are obviously prevalent in foods) typically have a high ozone demand and may consume the reactive species of ozone breakdown before they can reach the microorganisms. The second reason is that the surfaces of foods (where most of the reactive species are consumed) may have folds and crevices that "hide" the microorganisms so that ozone cannot act upon them. Colonies of microorganisms may exist in multiple levels or reside under protective secretions. Therefore,

two conditions become very important. The first is that the ozone be maintained and well dispersed in solution for the longest duration possible so that the ozone has a greater opportunity to make its way into crevices and through multilevel colonies. This may be achieved through high stir speed (while the ozone is bubbled into the water) and restricted headspace escape from the vessel. The second is to minimize the degree to which the microorganisms "hide" on the surfaces of foods.

Justification

Minimally processed foods, such as vegetable sprouts, can be potentially harmful if they carry significant populations of pathogenic bacteria. Chlorine is commonly used as a disinfectant to wash produce and processing equipment. Chlorine has been proven to be an efficient disinfectant, producing a two-log reduction in the amounts of microorganisms present on produce (Adams et al., 1989). Chlorine also has been shown to produce potentially toxic by-products, such as trihalomethane compounds (Graham, 1997). Minimally processed foods would benefit from a safer, more effective antimicrobial treatment such as ozonation.

Objectives

1. Determine the effect of varying ozone concentrations on the microbial populations of alfalfa sprouts.
2. Determine the best method of application of ozone to reduce the microbial populations of alfalfa sprouts.
3. Develop an ozone treatment of alfalfa sprouts, which will decrease the microbial load of the sprouts so as not to exceed a maximum microbial count of 10^6 cfu per gram, and is free of pathogens.

Project Outline

The first stage of this project involved the evaluation of treatment conditions for the application of ozone. Variations in several conditions were evaluated, including temperature, treatment vessel, stir speed and ozone application method. In the initial phase of the project, damage to the sprouts was evaluated along with enumeration of a control sample, treated sample and rinsed sample.

The second stage of the project involved: determination of the microbial counts on the

seeds, what level of reduction could be achieved in the seeds and whether this reduction would persist over the duration of the growing period. Also, in an attempt to maintain the reduction in the treated seeds, the water used for watering the sprouts twice daily was ozonated prior to use. The final component of the second stage involved the determination of the microbial counts on the seeds for each of the days that the sprouts are grown for a variety of treatments.

The final stage of the project involved reevaluation of the optimal treatment conditions including watering with ozonated water, end rinsing with both water and ozonated water, and prolonged exposure to ozonated water while being gently stirred.

Methods

Seed Sprouting

The alfalfa seeds used were purchased in 4-oz packages. They are distributed by NK Lawn & Garden and are meant for use in home sprouting and contain no additives such as antifungal agents or fertilizers. The seeds were grown in a sprouting apparatus also made and distributed by NK Lawn & Garden. The sprouter consists of three sprouting trays a top water reservoir and a bottom water reservoir. Approximately 5 g of seed where placed in each sprouting tray used. The sprouts were watered as indicated in the instructions that came with the sprouter, twice daily with 300 ml of autoclaved, deionized water at 23 °C (unless otherwise dictated by the specific procedure). The sprouts were grown at room temperature (23 °C) for a period of 4 days as indicated by the instructions. They were grown in indirect light. All growing procedures followed this general outline unless otherwise specified.

Treatment Conditions (Ozonation and Rinsing)

Ozone was produced using an ozone generator, LT #1 ElectroChemical Ozone Generator manufactured by Lynntech, Inc., and applied to the sprouts as specified by each procedure. Sprouts were treated with ozonated water at various concentrations and in a variety of ways to determine the best method of application. The water used in all applications was deionized and autoclaved. The first treatment vessel used was simply a 600-ml beaker, which was covered with aluminum foil. The beaker held a 12 g sample of sprouts and 400 ml of autoclaved, deionized water. After two trials, a stoppered 500-ml flask was used. Through one hole in the

stopper was the inlet with an attached sparger. Through the other was the headspace outlet which escaped through a small diameter tube. The treatment vessel contained 12 g of sprouts and 400 ml of autoclaved, deionized water. The sample and water was agitated using a magnetic stirrer.

Rinsing the sprouts was accomplished by placing the sample in a container containing 400 g of autoclaved, deionized water and agitating the sample with a magnetic stir bar for 5 minutes. This process was intended to simulate washing the sprouts as would be performed in the home or during production.

Soaking the seeds consisted of a 4-hour period of the seeds setting in 200 ml of autoclaved, deionized water.

When the ozone treatment was performed by stirring as specified in the latter trials, the sprouts were placed in autoclaved, deionized, ozonated water (400 ml in a 600 ml beaker) and gently stirred for a period of 20 minutes using a magnetic stir bar.

Measurement of Ozone

Ozone concentration (residual) was measured using the indigo method as developed by Bader and Hoigne (1981). The principal of this method is based on the activity of ozone across a double bond of a sulfonated indigo dye, resulting in a decolorization which can be measured using a spectrophotometer measuring at 600nm wavelength. The change in absorbance can be used to calculate the amount of ozone present (ppm). For the purpose of this experiment, the residual ozone was measured. The residual ozone is that ozone which remains immediately after the treatment has been performed. The calculation of ozone is as follows.

O_3 in ppm = dA*100 / f*b*V Where,

dA = difference in absorbance between sample and blank

b = path length of cuvette in cm

V = volume of ozone water sample added to the cuvette in mL

f = 0.42

Microbiological analysis

Non-selective plate count agar (PCA) was used to determine the populations of aerobic microorganisms. A 10 g sample of the sprouts or seeds was prepared with 90 ml of peptone water (0.1% peptone concentration) and stomached for 1-2 minutes. Serial dilutions were prepared of the liquid contents of the stomached sample. From the appropriate dilutions, spread plates were prepared and incubated at 37 °C for 2 days.

Physical analysis

The quality of the ozone-treated sprouts was determined by examining the sprouts under the microscope. The integrity of the sprout was examined along with the presence or absence of surface damage. The color and texture of the sample sprouts was evaluated in a comparative sense to the control sample.

Results and Discussion

Rinsing Versus End Treatment

Following the procedure as described in the growing instructions that came with the seed sprouter; sample seeds were grown for a period of 4 days. A 12 g sample of sprouts was placed in a 600 ml beaker containing 400 ml of water (23 °C). The ozone was bubbled into the water with the aid of a sparger and the contents of the beaker were stirred with a magnetic stirrer. The ozone was applied for a period of 5 minutes. After ozonation, the sprouts were removed from the water and a 10 g sample was taken for microbial analysis. The results of this evaluation was that the count on the rinsed, untreated sample was 7.0×10^8 cfu/g and the count for the treated sample was 5.2×10^7 cfu/g (Figure 1). The ozone treatment produced a 1.18 log reduction. Using this experiment as a basis, other variations on treatment conditions were tested.

For the second trial, the temperature of the water used for treatment of the sprouts was 4 °C rather than 23 °C. All other conditions were identical. The results of this evaluation was that the counts on the rinsed, untreated sample was 8.0×10^8 cfu/g and the count for the treated sample was 4.4×10^7 cfu/g (Figure 1). These findings suggest that the temperature of the treatment water does not significantly effect the ability of the ozone to inactivate the microorganisms (within the tested range) compared to the previous trial. Two opposing

activities occur as the temperature of the water containing the ozone is increased. At lower temperatures, ozone is more soluble in water than at higher temperatures. However, at higher temperatures, the breakdown of ozone is more rapid. Therefore, while more ozone is present in colder water its oxidizing activity is reduced. Opposing finding occur in published research as well. Katzenelson et al. (1973) reported that a slight change in the temperature of the water used to carry the ozone had no effect on the inactivation kinetics of ozone. Herbold et al. (1989) reported that the effectiveness of ozone on *E. coli* was reduced as the temperature of the carrier water is increased.

In an attempt to increase the residual time of the ozone in the water, a flask with a restricted headspace escape was used (as described in the methods section). The results of this trial (#3) was that the counts on the rinsed, untreated sample was 2.5×10^8 cfu/g and the count for the treated sample was 2.6×10^7 cfu/g (Figure 1). At these conditions, the ozone-induced reduction was 0.99 log. These findings suggest that the conditions of the new treatment vessel did not significantly effect the ability of the ozone to inactivate the microorganisms. Either the restricted flow was ineffective in increasing the residual time of the ozone in solution or the increased residual time was ineffective in killing more microorganisms. However, this treatment vessel was convenient to use and was therefore used throughout the remainder of the trials.

A non-rinsed sample was used as the control comparison for each respective treatment. For trial 4, two samples were taken from the same batch and were not rinsed before counts were made. Two samples were taken from the same batch and were rinsed prior to counts. Two samples were taken from the same tray and were treated by bubbling ozone (5 minutes) after the prior to counts. The results of this evaluation was that the counts on the unrinsed, untreated sample were 2.3×10^9 cfu/g and 1.4×10^9 cfu/g. The counts on the rinsed, untreated sample were 3.0×10^8 cfu/g and 1.5×10^8 cfu/g. The counts for the treated sample were 2.7×10^7 cfu/g and 2.5×10^7 cfu/g. Taking the average of these counts, the reduction produced from a rinsing process alone was 0.96 log. The treated sample produced a greater reduction, 1.93 log (Figure 2).

Application of Ozone in an Intense Rinsing Procedure

In an attempt to increase the likelihood of the ozone contacting microorganism colonies, the sprouts were stomached for a time of 2 minutes in the presence of ozonated water. Two samples were taken from the same tray for use as control and treated samples. The results of this evaluation was that the counts on the rinsed, untreated sample was 9.2×10^7 cfu/g and 2.1×10^8 cfu/g, and the count for the treated sample was 2.6×10^7 cfu/g and 4.9×10^7 cfu/g respectively (Figure 3). The process of stomaching the sample in the presence of ozonated water, which was very destructive to the sprouts, was ineffective in producing a greater reduction in the microbiological populations, a 0.81 log reduction was determined from this treatment. Such a process is too intense on the sprouts for practical use, but this trial was an indication of the difficulty that is present in getting the ozone to act upon the target microorganisms. The previous treatments were somewhat detrimental to the conditions of the sprouts. The color of the sprouts was brighter for the treated samples compared to the control and the crispiness of the sprouts was somewhat compromised following the ozone treatment.

Pretreatment Process

A previous trial had shown that the microbiological counts on the seeds prior to sprouting was around 10^5 cfu/g. Considering this a great place to decrease the microbial load of the seeds, a pretreatment of the seeds was performed in trial 6. The pretreatment consisted of a 4-hour period of soaking the seeds prior to the placement of the seeds in the growing trays. The purpose of this period was to allow the inactive, desiccated microorganisms present to reactivate and therefore be more susceptible to the ozone attack. It has been reported that desiccated microorganisms are much more resistant to the attack of the ozone. The soaking period was allowed to be only 4 hours so that the amount of cell clustering and protective coating secretions were limited. Using the indigo method for determination of residual ozone in the treatment solution, it was determined that the residual ozone in the treatment of the seeds (referred to as the pretreatment) was 12 ppm. Following full growth, the five-minute ozone treatment period on the sprouts produced residual ozone of 9 ppm. The resulting counts on the pretreated seeds compared to the non pretreated seeds showed that the treatment was ineffective in producing a significant reduction (0.13 log), producing counts of 2.4×10^9 cfu/g and 1.1×10^9 for the non-

treated and treated sprouts respectively. It seems that if the pretreatment is effective in initially reducing the microbial populations, this advantage is lost over the growing period of the sprouts. The coupling of the pretreatment and end treatment produced an identical count compared to the use of an end treatment alone (2.4×10^7 cfu/g for both the non-pretreated and pretreated sprouts respectively). The reduction obtained from these two processes was 2.00 log. The microbial counts on the seeds was determined to be 3.2×10^5 cfu/g for the non pretreated sample and was estimated to be 10^3 cfu/g for the pretreated sample (Figure 4).

Trial 7 was performed to evaluate the ozone treatment compared to an intensive rinsing treatment. The amount of microorganisms that were present in water (after rising) that was used to rinse the sprouts was evaluated. In addition to this, the application of ozone in the stomaching bag was also reevaluated. A third factor tested was that of a reduced ozone supply. Due to the damage seen in the sprouts given the previous treatments, the rate setting on the ozone generator was reduced so that the amount of ozone supplied to the treatment vessel would be reduced and the damage of the sprouts would be ideally lessened.

The control count for this trial was very high compared to previous trials (1.1×10^{10} cfu/g, about half a log higher). In this experiment, the pretreated ozonated seeds had a lower final count by about half a log compared to the control, possible due to the high control count that was obtained. Although the pretreated sample produced a count that was 0.53 log lower than the control, the difference was not statistically significant. The process of stomaching the sprouts in ozonated water resulted in a reduction of 0.98 log compared to the control (Figure 5). This process was less effective than treating the sprouts by direct bubbling of ozone into water, which produced a reduction of 1.93 log (compared to the control). It was interesting to see that the intense rinsing process was equally effective in reducing the counts as was the ozone treatment, producing a reduction of 2.10 log (compared to the control). Further studies are needed to confirm these findings. Counts on the rinse water showed that most of the removable microorganisms are removed in the first wash (10^7 cfu/g) and following washes are comparatively ineffective (10^5 cfu/g for the third wash). In general the results of this study follow others in that the reduction obtained by the ozone treatment was about 2 log. Damage to the sprouts was somewhat lessened by the use of lower rate setting on the ozone generator. The residual ozone in the pretreatment of the seeds was 11 ppm, similar to that measured before.

The residual ozone in the water used for the stomached sample was 14 ppm. The residual after the end treatment was only 3.1, indicating that either less ozone was present during the end treatment or it was consumed faster than seen before. Both conditions may be present, especially since the rate on the ozone generator was decreased. So, while the reduction of the generator rate had a positive effect on the quality of the seeds, it may have also contributed to the comparatively high counts measured in the mature sprouts.

Growth Behavior Following Pretreatment

For the next experiment (trial 8), the population growth was studied over the four-day growing period. Each tray was watered twice daily and separately, so as not to contaminate the lower levels with the drained water from the preceding tray. Iodine values were used to determine the residual ozone. The ozonation time for the pretreatment seeds was 5 minutes. The ozonation time for the sprouts was 2.5 minutes, so as to reduce the damage caused by the treatment. The residual ozone for the seed treatment had increased to about 25 for all samples. The residual ozone for the end treatment was about 15, also an increase from that seen in previous trials.

The microbial counts on the seeds were determined to be 1.8×10^5 cfu/g for the non-pretreated sample and 1.7×10^4 cfu/g for the pretreated sample. The ozone pretreatment was effective in reducing the seed flora by 0.99 log. This reduction was shown to be overcome in the latter growth of the natural flora. After 1 day of growth, the pretreated seeds had a count of 1.5×10^7 cfu/g, whereas the count on the control was greater than 10^8 cfu/g (est.). The pretreated sprouts were 2.8×10^8 cfu/g after two days after which they reached a plateau at about 10^8 cfu/g. The non-pretreated sprouts reached a plateau seemingly earlier and at about 10^9 cfu/g (est.). Thought the dilutions used in this experiment were not adequate in viewing the entire picture of the population growth over the four-day period and the statistics were marginal, much information was gathered. Most of the growth of the natural flora occurs in the first two days after setting. A treatment should be developed to target this time before the bacterial cells become well incorporated onto the sprout surface. The counts on the pretreated sprouts increased by about 3 log after just one day (Figure 6).

In order to target the initial growth of organisms, an intense treatment of the sprout seeds

was developed and tested in trial 9. After the initial 4-hour soaking, the seeds were subjected to an ozonation treatment for 10 minutes. The seeds were placed in their trays and drained. After a 4-hour setting period they were again subjected to the same ozone treatment. After a third 4-hour setting period, the seeds were similarly ozonated, for a total of three treatments within 12 hours of the beginning of the soaking period. The residual ozone measurements in the treatments were 16, 15 and 35 ppm respectively. A 0.97 log reduction was obtained in the initial ozone treatment of the seeds (Figure 7). After 12 hours (after the third treatment) the treated seeds had a count of 1.0×10^5 cfu/g, whereas the non-treated sprouts had a count of 3.3×10^7 cfu/g. After the entire pretreatment, the treated seeds had a count that was 2.10 log lower than the non-treated seeds. After 36 hours (1 day after the third treatment) the microbial load was undetermined at a dilution of 10^{-6}, whereas the non-treated sprouts were determined to be 1.8×10^9 cfu/g. After the full 4-day growth, the counts of the pretreated and non-pretreated seeds were nearly identical (5.2×10^9 cfu/g for both the non-pretreated and pretreated sprouts). An end ozone treatment (17ppm residual) on the non-pretreated sprouts produced a 1.54 log reduction (compared to the control). An end ozone treatment on the pretreated sprouts (21 ppm residual) produced a 1.99 log reduction. The problem remains in that the intense ozone treatment initially is effective in maintaining low populations initially, but this effect is overcome with time and by the fourth day, the population has increased to be similar to the control. In addition to this, the treatment proved to be too harsh and the growth of the sprouts was inhibited.

In trial 10, when the treatment conditions were made less intense (first two treatments were for 5 minutes and the last treatment was 2.5 minutes) the similar results were observed (Figure 8). Ozonated water was used to water the sprouts twice daily, but was ineffective in maintaining lowered microbial populations. The less intense pretreatment did not cause the damage to the sprouts as seen in the previous pretreatment, but was insufficient in maintaining the desired reduction. The standard deviations for three of the conditions were very high due to one outlying data point in each condition.

Ozone Treatment by Using Pretreated Water

Two final studies (trial 11 and 12) were conducted in order to test methods which were intended to cause less stress to the sprouts while still producing the same observed kill. Results

of trial 7 indicated that an increased rinsing treatment might be beneficial in reducing the natural flora of the sprouts. Coupling this with an ozone treatment may give the desired reduction while causing less stress to the sprouts. Two control samples were compared to two ozone treatment samples consisting of bubbling the ozone into the suspended sprouts, and two ozone treatment samples consisting of gently stirring the sprouts in ozonated water for a period of 20 minutes.

The counts obtained from the control sample were 9.4×10^9 cfu/g and 2.7×10^9 cfu/g. An end treatment consisting of bubbling ozone into the sample (2.5 minutes) produced sprouts with a 1.76 and 2.08 log reduction relative to the respective control. When the sprouts were placed in pre-ozonated water (30-32 ppm) and stirred for 20 minutes, the reductions obtained were 1.70 and 1.51 log. The stirring method is less intense than the direct bubbling method. Although better reductions can be obtained using direct bubbling, the damage to the sprouts would be considered unacceptable. While the 2.5 minute treatment was not as damaging to the sprouts as the 5 minute treatment, they were not as crisp as the stir-treated sprouts. Therefore, this method of treatment seems to be a viable alternative to the bubbling and stirring treatment. The advantage to this treatment is that the damage to the sprouts is reduced. Stirred sprouts are much crisper in texture and show less breakage than ozone bubbled sprouts.

Conclusions

Several conclusions can be made form the data obtained in this study.

1. Ozone is effective in reducing the natural flora on alfalfa sprouts by about 2 log, or to about 10^7 cfu/g. Though not investigated in this study, chlorine has been reported to produce a similar 2-log reduction. Therefore, ozone is a viable alternative to chlorine in the reduction of the natural flora of alfalfa sprouts prior to retail sale.
2. There are three limitations of ozone use in this application. First, the effect of ozone is limited to the ability of the ozone to penetrate into the surface topography of the sprout. The hydrophobic nature of plant surfaces and the high rate of consumption of ozone and its oxidative products create limitations to this penetration. Second, the presence of organic matter will consume the ozone, thereby reducing its effectiveness. For optimization of this process, a continuous process may be promising since loose organic matter (seed coats and suspended matter) can be removed form the aqueous media via filtration. The third limitation is that increased ozone tends to damage the sprouts, particularly the crispiness, which is a major attribute of this product.
3. The most effective process tends to damage the sprouts. Therefore, with the product integrity in mind, the best process would be treatment via addition and subsequent stirring of the sprouts in ozonated water for an adequate amount of time, 20 minutes in this study.
4. It would be interesting to see the effect of a sonication treatment on the seeds or sprouts and whether this treatment would aid in the disruption of colonies or dislodge the microorganisms from their protective locations, thereby facilitating a greater kill.

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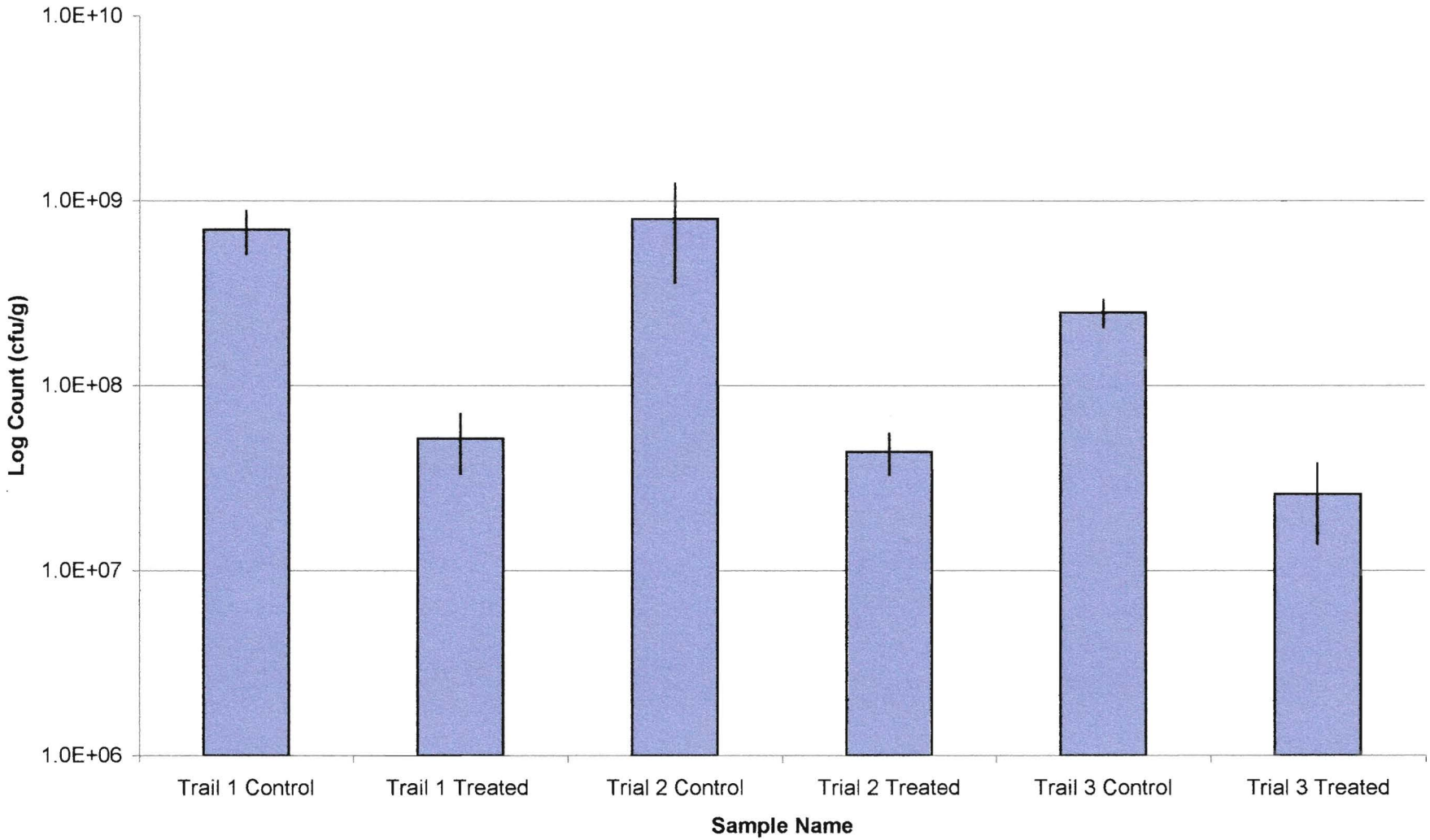

Figure 1. Total aerobic plate counts obtained form the first three trials. All control samples were rinsed control samples. The trial 2 treated sample differed from trial 1 in that cold water was used. The trial 3 treated sample differed from trial 1 in that the described treatment vessel was used. For each condition, about 1 log reduction was observed.

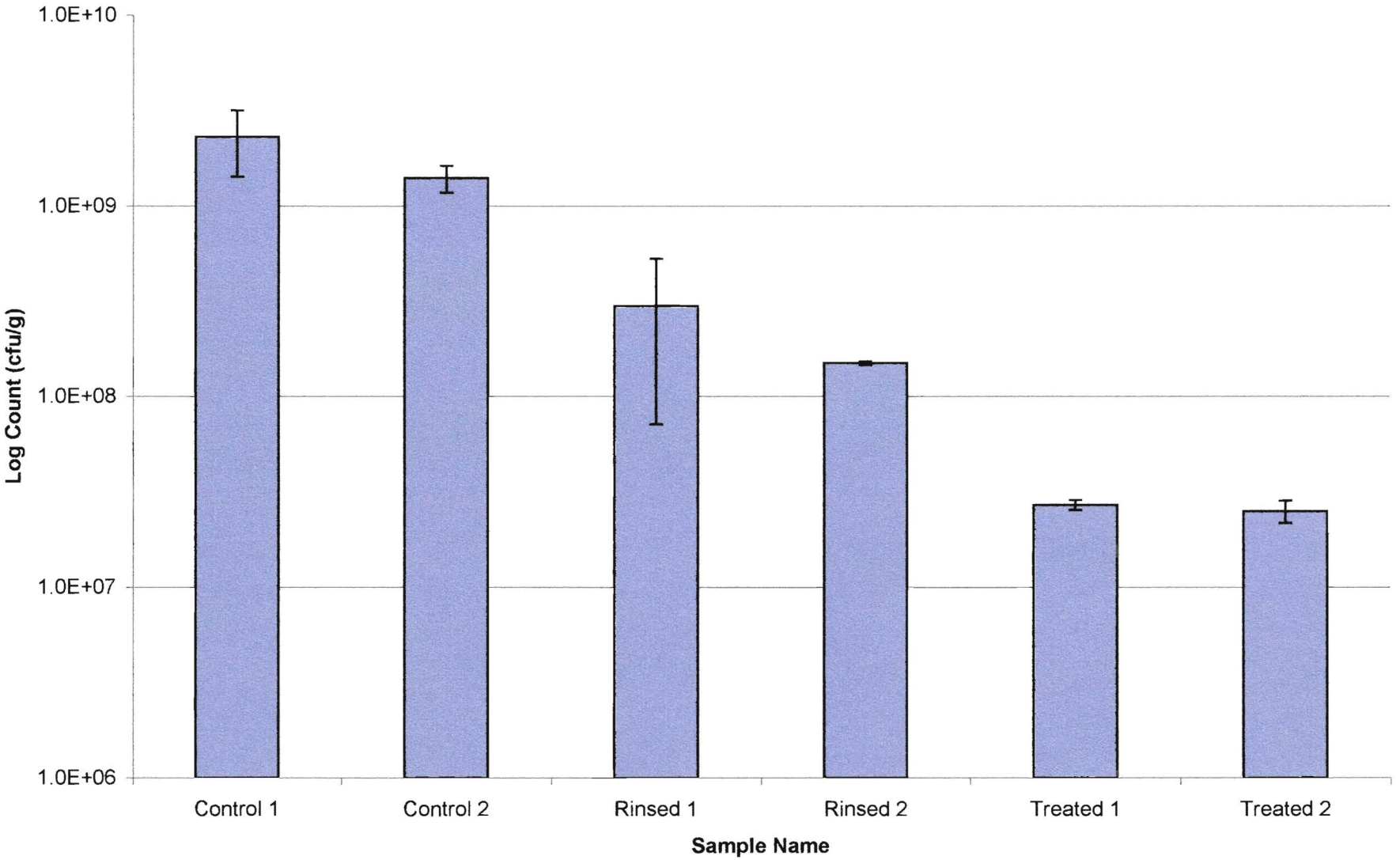

Figure 2. Total aerobic plate counts obtained from the fifth trial. The control samples were not rinsed prior to counts. The rinsed samples were prepared according to the description in the methods section. The treated samples were subjected to a five minute ozonation by bubbling. Rinsing the sprouts produced a 1 log reduction, whereas the ozone treatment produced a 2 log reduction.

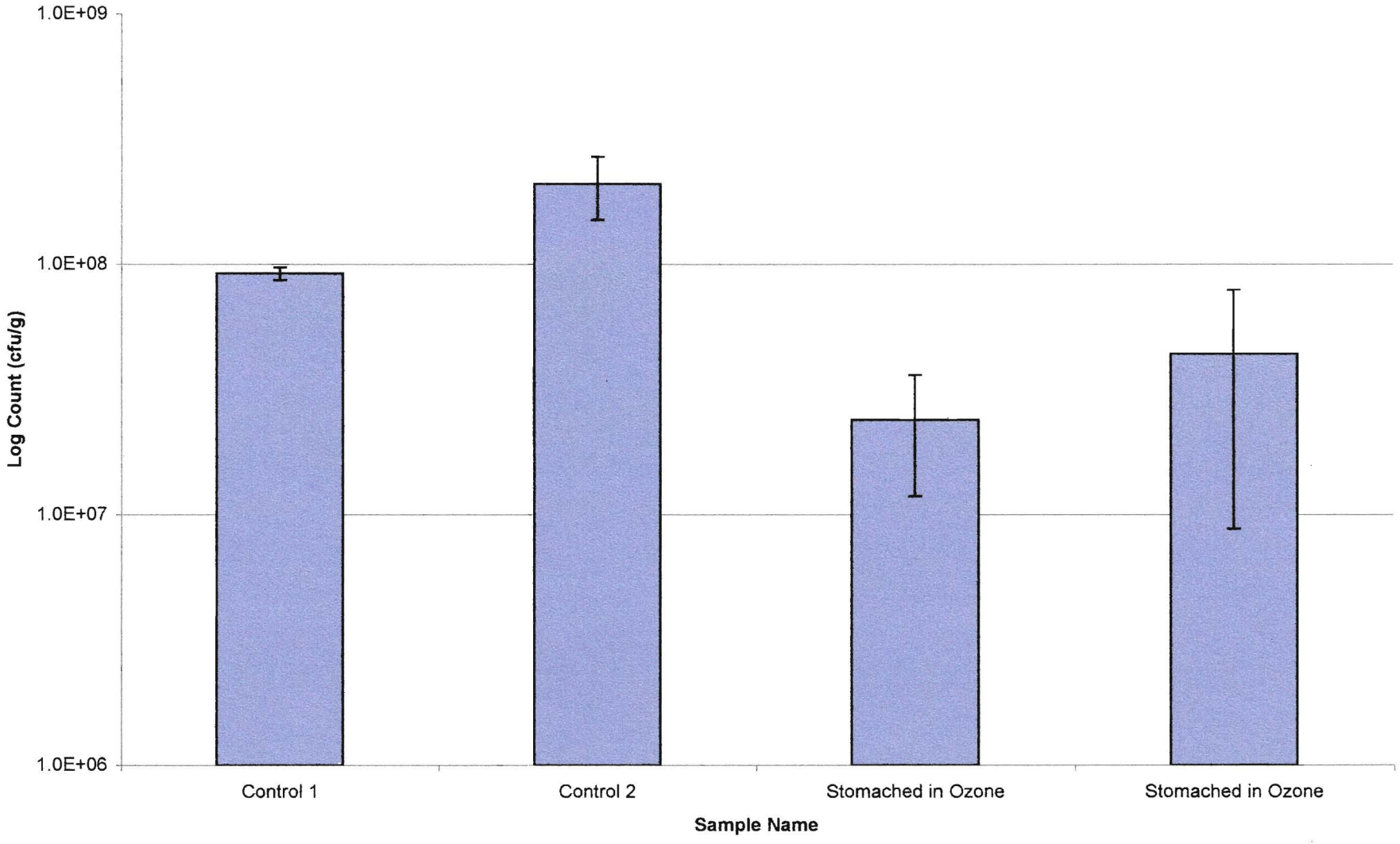

Figure 3. Total aerobic plate counts obtained form the fourth trial. All control samples were rinsed control samples. The treated samples were stomached in the presence of ozonated water. This method of ozone application was no better than the methods previously tried and again showed about a 1 log reduction.

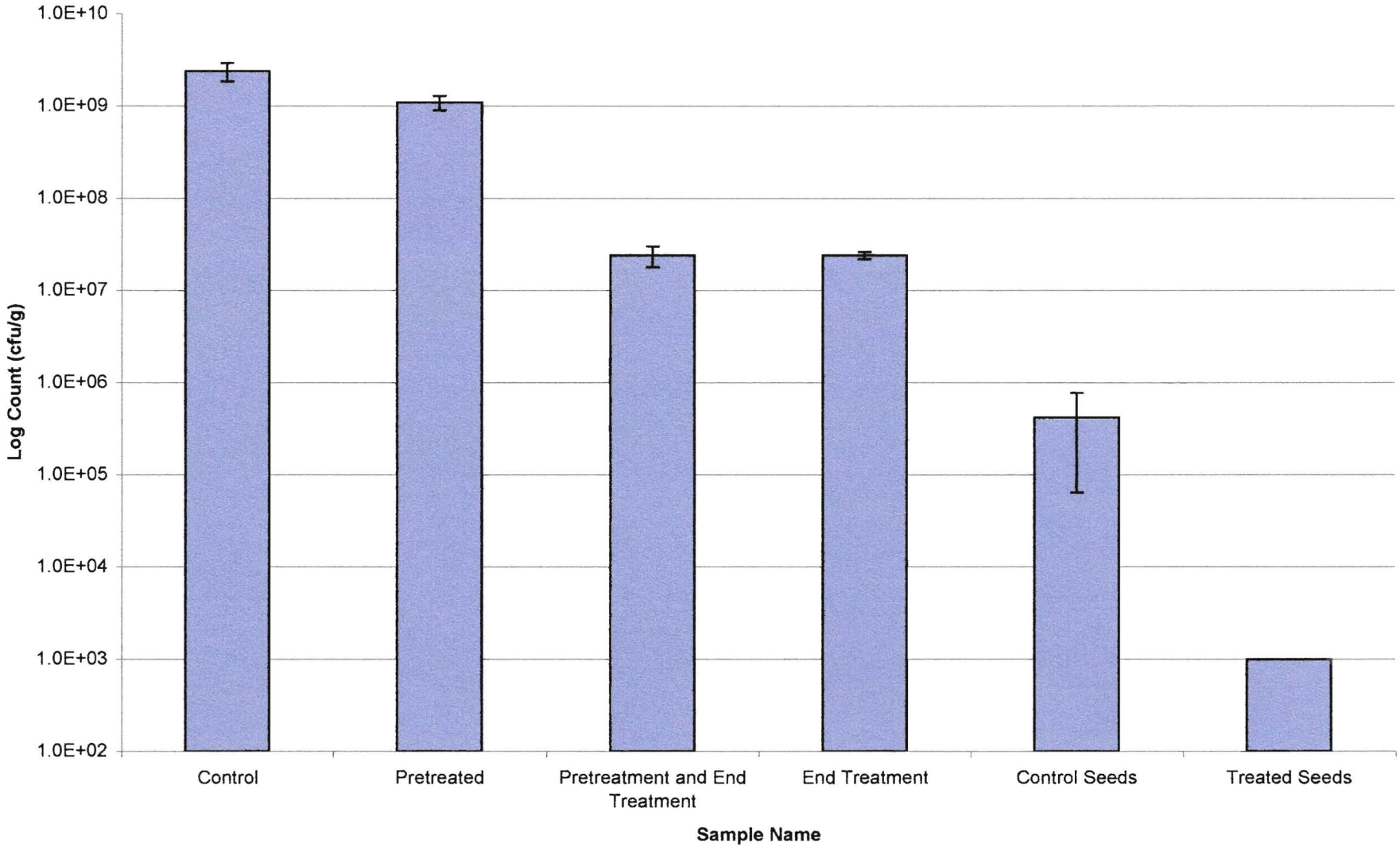

Figure 4. Total aerobic plate counts obtained from the sixth trial. The control sample was not rinsed prior to counts. By the end of the growing period, the pretreatment proved to be inneffective. The combined end treatment and pretreatment was equivalent to the end treatment alone. It was determined that the seeds have an initial microbial load of 105 cfu/g. The pretreatment was effective in reducing this initial load, but to what degree was undetermined in this trial.

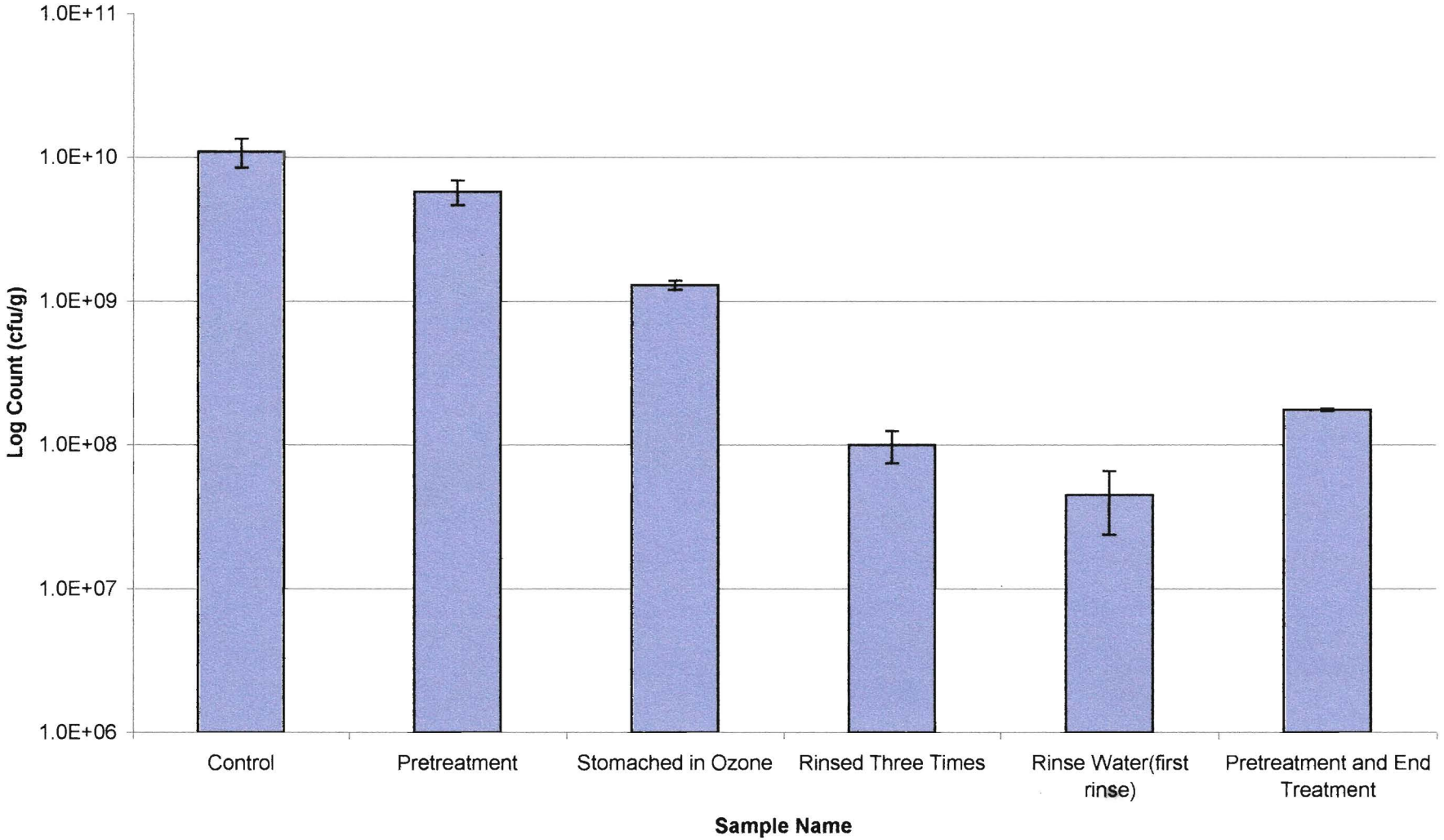

Figure 5. Total aerobic plate counts obtained from the seventh trial. The control sample was not rinsed prior to counts. The pretreatment was shown to produce sprouts with a final aerobic plate count approximately one half log lower than the control. The stomaching process of ozone application was determined to be effective in reducing the final counts by about 1 log. Rinsing the sprouts, as described in the methods section, was effective in reducing the counts by about 2 log, similar to the end ozone treatment.

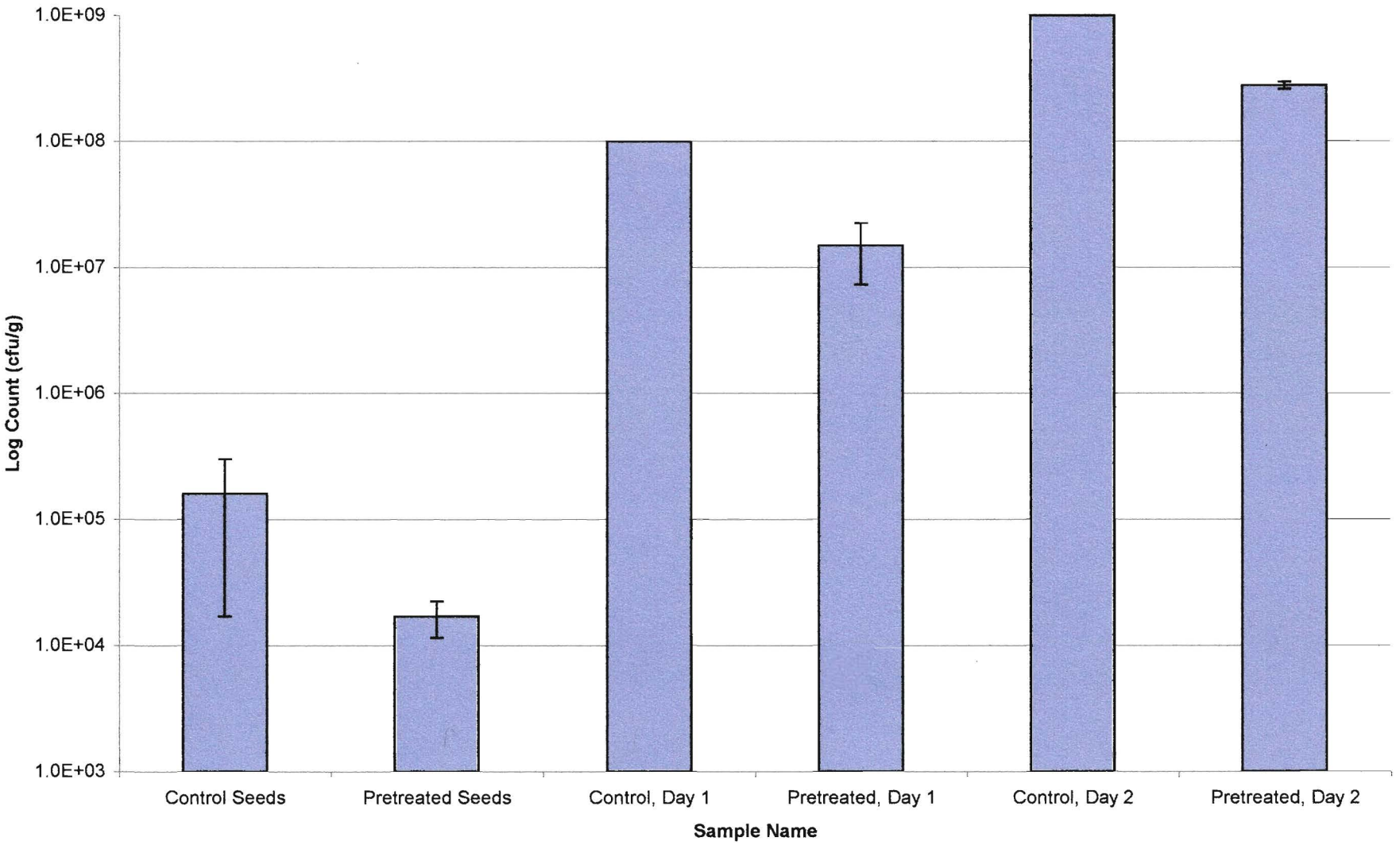

Figure 6. Total aerobic plate counts obtained from the eighth trial. The control sample was not rinsed prior to counts. The first treated sample was only pretreated. Each sample was allowed to grow for a period of 4 days, counts of the first two days are listed. This firgure better shows that the natural flora can grow rapidly on the sprouts after the ozone pretreatment.

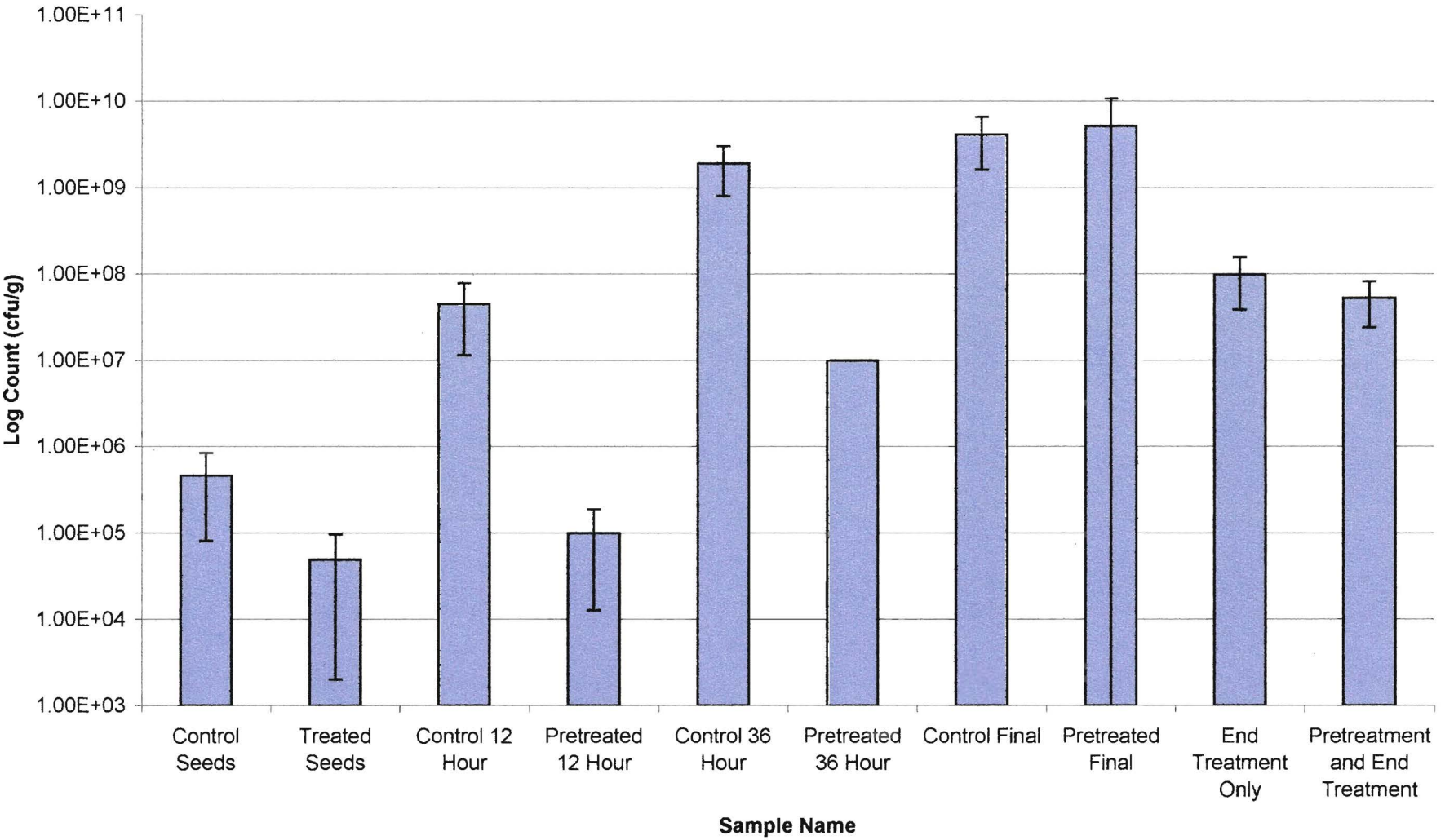

Figure 7. Total aerobic plate counts obtained from the ninth trial. The control sample was not rinsed prior to counts. Both the control sample and the pretreated sample were allowed to grow freely for a period of 4 days, counts of the first 36 hours are shown. The pretreatment for trial 9 was more intense that that used in trial 8 and was effective in keeping the microbial load low relative to the control. After the full growing period, however, the counts on the control and pretreated samples were similar.

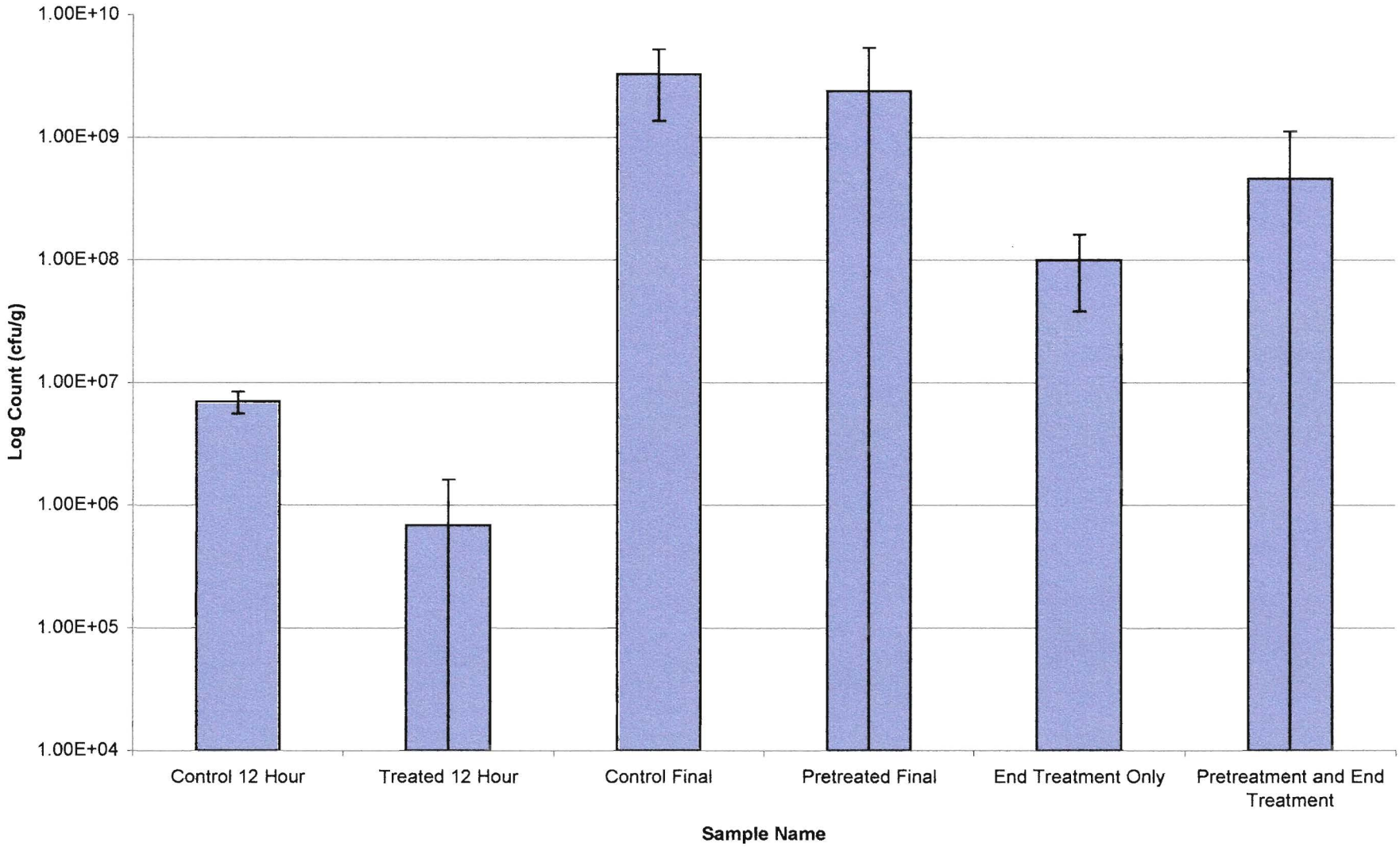

Figure 8. Total aerobic plate counts obtained from the tenth trial. The control sample was not rinsed prior to counts. Counts at 12 hours show that the pretreated sample is still about 1 log lower, however, this is overcome in the remaining growth period. Two other treatments showed typical success in reducing the plate counts.

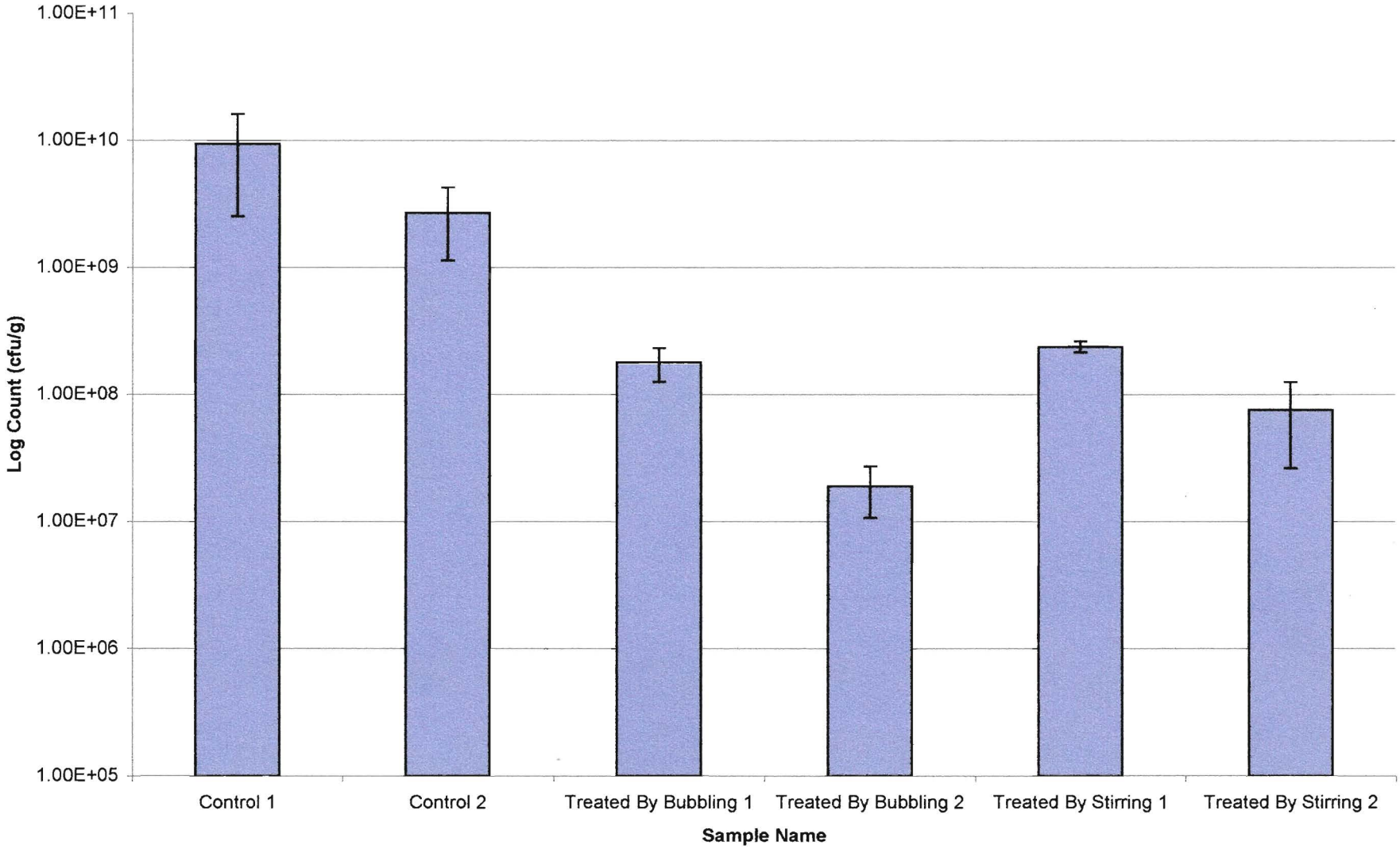

Figure 9. Total aerobic plate counts obtained from the final two trials. The control samples were not rinsed prior to counts. The process of bubbling ozone into the aqueous-suspended sprouts was slightly more effective than the process of stirring the sprouts in ozonated water.

Figure 10. Sprouting apparatus used for growth of sprouts.

Figure 11. Treatment vessel used to add ozone by bubbling into water.

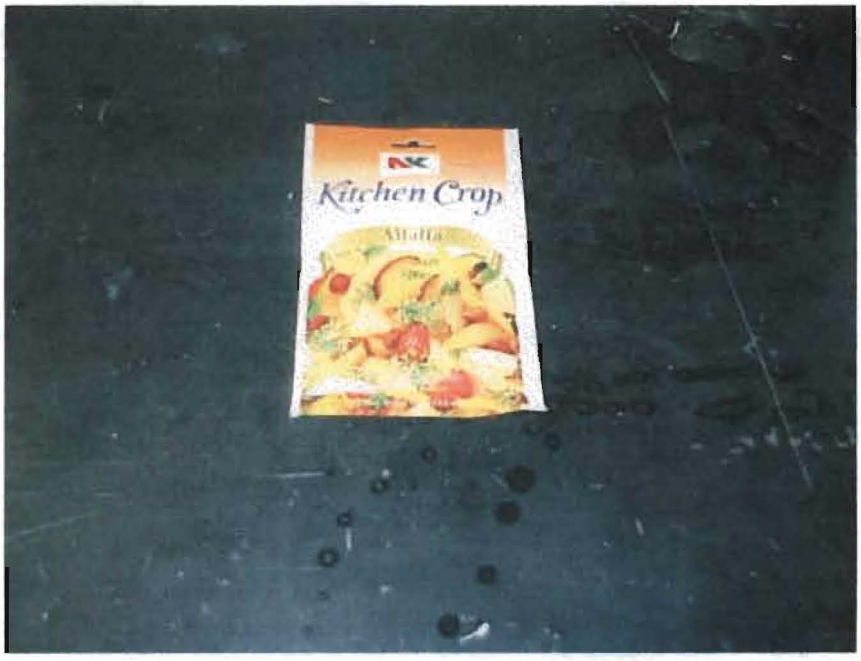

Figure 12. Retail seeds used for this study.

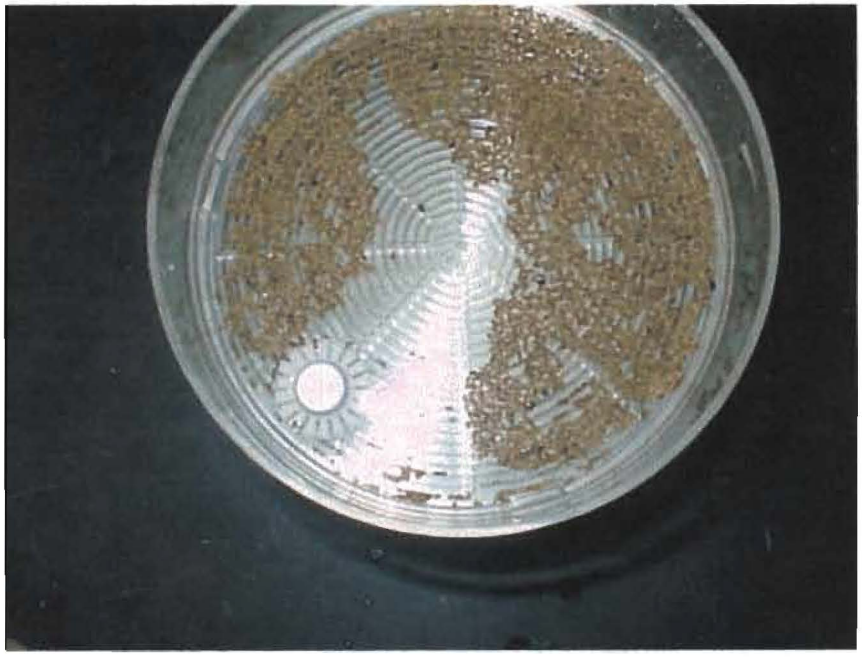

Figure 13. Seeds after receiving the first watering.

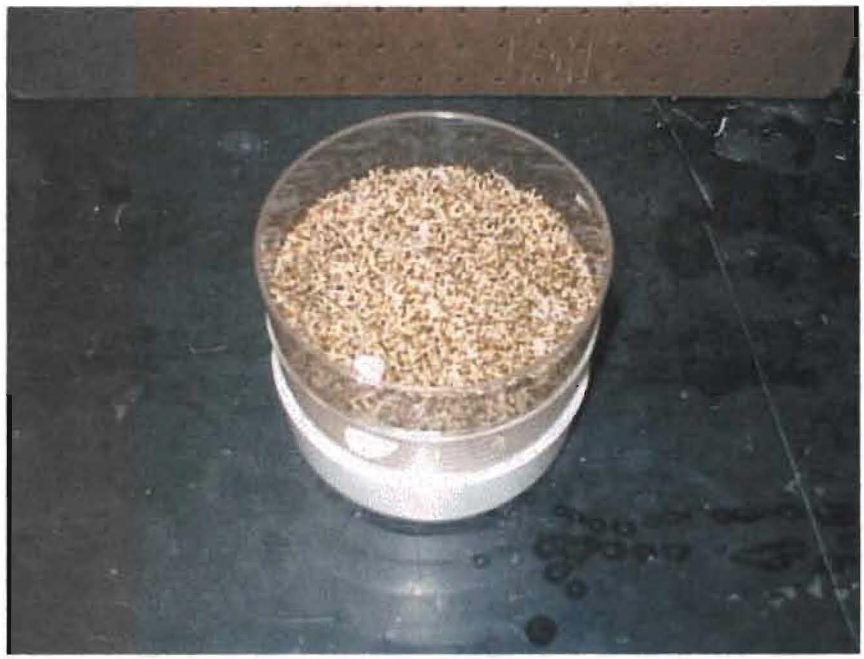

Figure 14. Sprouts after 36 hours of growth.

Data Sheet Summary

Trail #	Condition	Aerobic Count	Description	Reduction	Residual Ozone
1	Control	7.0×10^8	Control, Rinsed		
	Treated	5.2×10^7	Treated, Five Minutes	1.18 log	
2	Control	8.0×10^8	Control, Rinsed		
	Treated	4.4×10^7	Treated, Five Minutes, Cold Water	1.19 log	
3	Control	2.5×10^8	Control, Rinsed		
	Treated	2.6×10^7	Treated, Five Minutes, Treatment Flask	0.99 log	
4	Control	9.2×10^7	Control, Rinsed		
	Control	2.1×10^8	Control, Rinsed		
	Treated	2.4×10^7	Treated, Stomached in Ozonated Water for 2 Minutes	0.81	
	Treated	4.4×10^7	Treated, Stomached in Ozonated Water for 2 Minutes		
5	Control	2.3×10^9	Control, Non Rinsed		
	Control	1.4×10^9	Control, Non Rinsed		
	Washed	3.0×10^8	Control, Rinsed	0.96 log	
	Washed	1.5×10^8	Control, Rinsed		
	Treated	2.7×10^7	Treated, Five Minutes, Treatment Flask	1.93 log	
	Treated	2.5×10^7	Treated, Five Minutes, Treatment Flask		
6	Control	2.4×10^9	Control, Non Rinsed		
	Treated	1.1×10^9	PreTreated, Five Minutes after Soaking	0.13 log	12 ppm
	Treated	2.4×10^7	PreTreated, Five Minutes after Soaking, End Treatment	2.00 log	12 ppm / 9 ppm
	Treated	2.4×10^7	End Treated, Five Minutes	2.00 log	9 ppm
	Control Seeds	4.2×10^5	NonTreated Seeds		
	Treated Seeds	10^3 est.	PreTreated Seeds		12 ppm
7	Control	1.1×10^{10}	Control, Non Rinsed		
	Treated	5.8×10^9	PreTreated, Five Minutes after Soaking	0.53 log	11 ppm
	Treated	1.3×10^9	Treated, Stomached in Ozonated Water for 2 Minutes	0.98 log	14 ppm
	Washed	1.0×10^8	Control, Rinsed 3 times	2.10 log	
	Wash Water	4.5×10^7	Microbiological Counts of First Wash Water		
	Treated	1.8×10^8	PreTreated, Five Minutes after Soaking, End Treatment	1.93 log	12 ppm / 3.1 ppm
8	Control Seeds	1.6×10^5	NonTreated Seeds		
	Treated Seeds	1.7×10^4	PreTreated Seeds	0.99 log	21 ppm
	Control 1 Day	$>10^8$	Control, Non Rinsed		
	Treated 1 Day	1.5×10^7	PreTreated, Five Minutes after Soaking		33 ppm
	Control 2 Day	$>10^9$	Control, Non Rinsed		
	Treated 2 Day	2.8×10^8	PreTreated, Five Minutes after Soaking		31 ppm
9	Control Seeds	4.6×10^5	NonTreated Seeds		
	Treated Seeds	4.9×10^4	PreTreated Seeds	0.97 log	16 ppm
	Control 12 Hour	4.5×10^7	Control, Non Rinsed		
	Treated 12 Hour	1.0×10^5	PreTreated, Five Minutes after Soaking	2.10 log	35 ppm
	Control 36 Hour	1.9×10^9	Control, Non Rinsed		
	Treated 36 Hour	$>10^7$	PreTreated, Five Minutes after Soaking		
	Control Final	5.2×10^9	Control, Non Rinsed		
	Treated Final	5.2×10^9	PreTreated, Five Minutes after Soaking		
	Treated	9.8×10^7	End Treated (using non pretreated sample), Five Minutes	1.54 log	17 ppm
	Treated	5.3×10^7	End Treated (pretreated sample), Five Minutes	1.99 log	21 ppm
10	Control 12 Hour	7.0×10^6	Control, Non Rinsed		
	Treated 12 Hour	6.9×10^5	PreTreated, Five Minutes after Soaking	1.10 log	19 ppm
	Control Final	3.3×10^9	Control, Non Rinsed		
	Treated Final	2.4×10^9	PreTreated, Five Minutes after Soaking		
	Treated	1.0×10^8	End Treated (using non pretreated sample), Five Minutes	1.23 log	17 ppm
	Treated	4.6×10^8	End Treated (pretreated sample), Five Minutes	0.87 log	18 ppm
11&12	Control	9.4×10^9	Control, Non Rinsed		
	Control	2.7×10^9	Control, Non Rinsed		
	Treated	1.8×10^8	Treated By Bubbling	1.76	19 ppm
	Treated	1.9×10^7	Treated By Bubbling	2.08	23 ppm
	Treated	2.4×10^8	Treated By Stirring	1.7	31 ppm
	Treated	7.6×10^7	Treated By Stirring	1.51	32 ppm